PCT

WORLD INTELLECTUAL
Interna....

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ :		(11) International Publication Number:	WO 96/03521
C12P 21/06	A1	(43) International Publication Date:	8 February 1996 (08.02.96)

(21) International Application Number: PCT/US95/08971

(22) International Filing Date: 21 July 1995 (21.07.95)

(30) Priority Data:
08/278,281   21 July 1994 (21.07.94)   US
Not furnished   20 July 1995 (20.07.95)   US

(71) Applicants: YISSUM RESEARCH AND DEVELOPMENT CO. [IL/IL]; Hebrew University of Jerusalem, P.O. Box 4279, 91042 Jerusalem (IL). LASTER, Morris, C. [US/IL]; 34 Derech Haboresh, 97278 Jerusalem (IL).

(72) Inventors: OPPENHEIM, Amos, B.; 5 Harav Shrem Yossef Street, 96920 Jerusalem (IL). GILADI, Hilla; 25 Hashalom Street, P.O. Box 570, 97706 Mevasseret Zion (IL). GOLD-ENBERG, Daniel; 9/1 Hakinor Street, 97500 Ma'ale Adu-mimn (IL). KOBI, Simi; 1/6 Anussey Mash'had Street, East Talpiyot, 93784 Jerusalem (IL). AZAR, Idit; 5/10 Hashom-rim Street, 76232 Rehovot (IL).

(74) Agents: LAVALLEYE, Jean-Paul et al.; Oblon, Spivak, McClelland, Maier & Neustadt, P.C., 4th floor, Crystal Square Five, 1755 Jefferson Davis Highway, Arlington, VA 22202 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: VECTORS AND TRANSFORMED HOST CELLS FOR RECOMBINANT PROTEIN PRODUCTION AT REDUCED TEMPERATURES

(57) Abstract

The invention relates to an expression vector, the nucleic acid sequence of which comprises a promoter that is capable of controlling, when the vector is in a bacterial host and the temperature is lowered to below about 20 °C, the production of recombinant peptide or protein encoded by a gene contained within the vector. The invention also relates to host cells containing the vectors and to a method of producing a recombinant protein.

Description

Vectors And Transformed Host Cells For Recombinant Protein Production At Reduced Temperatures

This application is a continuation in part of U.S. Serial No. 08/278,281, filed on July 21, 1994.

Technical Field

The invention relates to an expression vector, the nucleic acid sequence of which comprises a promoter that is capable of controlling, when the vector is in a bacterial host and the temperature is lowered to below about 20°C, the production of recombinant peptide or protein encoded by a gene contained within the vector. The invention also relates to transformed host cells containing the vectors, and to a method of producing a recombinant protein.

Background Art

Genetic engineering provides methods for the cloning of foreign genes, their cDNA sequences or portions thereof, and their introduction into bacterial host cells such as *Escherichia coli*. The level of production of a foreign protein in the host cell depends on appropriately arranged transcription and translation control sequences that permit the regulated expression of the desired proteins or peptides coded by the foreign genes, gene fragments, various gene fusions or mutants thereof.

Transcription (i.e., the process leading to the production of mRNA) is a key step in the expression of a gene. Transcription initiation occurs at specific promoters which regulate gene expression. Promoters are regulated negatively by repressors and positively by activator proteins. The initiation of transcription can be separated into several steps (Buc et al., Biochemistry (1985) 24:2712). In order to act effectively, promoters are made of three elements. A core sequence is recognized by RNA polymerase. This region is flanked by

the USR, or the upstream region, and the DSR, or the downstream region. The USR was shown to bind specific activator proteins (Adhya et al., Gene (1993) 132:1). Two modes of gene activation are generally considered. DNA-bound activator makes direct contact with RNA polymerase and thereby facilitates the binding and isomerization of RNA polymerase. Alternately, the bound activator changes the structure of the promoter, thereby favoring transcription initiation.

In order to maintain the desired foreign gene and to express it in a bacterial host cell at a high level for protein purification, it is essential to place the gene under the control of a regulated promoter that permits turning off of gene expression during the first stage of fermentation in which the cell mass is increased, and turning it on at the second fermentation stage in which maximal gene expression and protein production is needed.

Production of proteins utilizing a number of promoters and ribosome binding sites has been previously described. For example, the use of the pL promoter for regulated gene expression has been the subject of several references (e.g., Bernard et al., Gene (1970) 5:59; Derom et al., Gene (1982) 17:45; Gheysen et al., Gene (1982) 17:55; Hedgpeth et al., Mol. Gen. Genet. (1978) 163:197; Remaut et al., Gene (1981) 15:81; and Deryneck et al., Nature (1980) 287). A number of ribosome binding sites have been employed in order to obtain a high level of protein synthesis, such as the phage λ cII translation initiation region used for the production of cII (Oppenheim et al., J. Mol. Biol. (1982) 158:327; Shimatake et al., Nature (1981) 292:128). Similarly, production of human growth hormone (hGH) and bovine growth hormone (bGH) are described, inter alia, in U.S. Patent No. 4,997,916.

-3-

One of the major problems in producing eukaryotic
proteins in the bacterium *E. coli* is that the desired
protein is often obtained in inactive aggregates termed
inclusion bodies. The inclusion bodies can facilitate
protein purification provided that it is possible to
refold the desired peptide to the active protein form *in
vitro* by some process (see, for example, U.S. Patent No.
4,997,916). However, there are proteins for which *in
vitro* refolding is highly inefficient.

Several factors appear to contribute to the
formation of inclusion bodies. The most important of
these are the rapid synthesis of a single protein
following induction of gene expression in the
fermentation process, and the temperature at which
fermentation takes place. At high temperatures,
refolding of the individual molecule is slowed down. The
presence of a high concentration of incompletely folded
proteins leads to protein aggregation due to the inter-
molecular interactions of hydrophobic domains. In
production of the native protein, these hydrophobic
domains play an important role in the folding of the
protein into a fully active form.

At present, there is no known way to direct the
production of proteins in their native form during the
fermentation process. For some proteins (such as hGH and
bGH) it is possible to disaggregate and refold the
desired protein, while for others there is no known
refolding process. However, it is known that chaperonin
protein complexes can act to facilitate disaggregation *in
vivo*. This process can also be carried out on a small
scale in biochemical laboratories.

It has been shown that proteins expressed in *E. coli*
at low temperatures have an increased solubility (Schein
et al., Bio/Technology (1988) 6:291; Schein,
Bio/Technology (1989) 7:1141). For example, when

SUBSTITUTE SHEET (RULE 26)

interferon (IFN-α2) was expressed in a bacterial culture at 37°C, 95% of the protein was found in inclusion bodies. In contrast, only 27% of the protein was insoluble when the same culture was grown at 30°C. Similar results were obtained for IFN-γ, and Shirano et al. (FEBS (1990) 1:128) demonstrated the low-level expression of soluble lipoxygenease in *E. coli* at about 15°C.

Accordingly, there remains a need for vectors which allow for the isolation of biologically active eukaryotic protein from bacterial cells. It is an object of the present invention to provide such vectors, as well as methods for the production and isolation of eukaryotic proteins from bacterial cells in a biologically active form. It is another object of the present invention to provide host-vector systems for the production of a desired protein in native form.

These and other objects and advantages of the present invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

Disclosure of the Invention

The present invention provides an expression vector, the nucleic acid sequence of which comprises a promoter that is capable of controlling, when the vector is in a bacterial host and the temperature is lowered to below about 20°C, the production of recombinant peptide or protein encoded by a gene contained within the vector.

The invention also relates to transformed host cells containing the vectors, particularly transformed *E. coli* host cells.

In addition, the invention relates to a method of producing in native active form a desired protein encoded by a gene contained within the vector.

Brief Description of the Figures

Figure 1 depicts the DNA sequence of the pL promoter region including the nucleotides flanking the Eco RI and Bam HI sites. This sequence is also set forth in SEQ ID NO:1.

Figure 2 depicts the restriction map of plasmid pHG87, which contains the wild-type pL promoter.

Figure 3 depicts the restriction map of the plasmid pHG91, which contains the mutant pL promoter pL-9G-50. The sequence of this promoter is also set forth in SEQ ID NO:2.

Figure 4 depicts (A) the presence of the mutant pL promoter pL-9G-50 in the plasmid pHG91K and its insertion into phage λB299 by means of a double crossover recombination event and (B) the resulting vector lambdaA01107 (λA01107).

Figure 5 depicts the restriction map of the kanamycin resistant (Km^R) plasmid pIK86 which was employed in construction of expression vectors according to the invention.

Figure 6 depicts the restriction map of the plasmid pIK-EL, which contains the EL cspA promoter.

Figure 7 depicts the DNA sequence of the EL cspA promoter, which is comprised approximately of the nucleotides between the arrows labeled "EL". The sequence of the EL cspA promoter contained in pIK-EL is set forth in SEQ ID NO:3.

Figure 8 depicts the restriction map of the plasmid pIK-L, which contains the L cspA promoter.

Figure 9 depicts the DNA sequence of the L cspA promoter, which is comprised approximately of the nucleotides between the arrows labeled "L". The sequence of the L cspA promoter contained in pIK-L is set forth in SEQ ID NO:4.

Figure 10 depicts the restriction map of the plasmid pIK-M, which contains the M cspA promoter.

Figure 11 depicts the DNA sequence of the M cspA promoter, which is comprised approximately of the nucleotides between the arrows labeled "M". The sequence of the M cspA promoter contained in pIK-M is set forth in SEQ ID NO:5.

Figure 12 depicts the restriction map of the plasmid pIK-S, which contains the S cspA promoter.

Figure 13 depicts the DNA sequence of the S cspA promoter, which is comprised of the nucleotides between the arrows labeled "IS". The sequence of the ScspA promoter contained in pIK-S is set forth in SEQ ID NO:6.

Figure 14 depicts (A) the presence of the cspA promoter in the plasmid pIK86 and its insertion into phage λB299 by means of a double crossover recombination event and (B) the resulting vector lambdaPcspA (λPcspA).

Figure 15 depicts the temperature response of the pL-9G-50 promoter fused to a lacZ reporter gene and integrated in the bacterial chromosome in the presence (i.e., hip⁺, ●) and absence (i.e., hip⁻, O) of the positive regulator of the pL promoter, host integration factor.

Figure 16 depicts the temperature response of the pL-9G-50 promoter fused to a lacZ reporter gene and integrated in the bacterial chromosome in the presence (i.e., hip⁺, ●) and absence (i.e., hip⁻, O) of host integration factor. Cultures transferred from 37°C to about 15-16°C: (a) ß-gal levels, (b) cell density. Cultures transferred from about 15-16°C to 37°C: (e) ß-gal levels, (d) cell density.

Figure 17 depicts the expression of lacz under the control of various cspA promoters at about 15-16°C: (□) endogenous cspA mRNA; (●) EL-lacZ mRNA; (■) M-lacZ mRNA; (▲) S-lacZ mRNA.

Figure 18 depicts a partial restriction map of plasmid pHG309 (a derivative of plasmid pHG87) and pL-plac (which responds to the lac repressor and is enhanced by pL promoter elements).

Figure 19 depicts the expression of lacZ, in terms of enzyme activities and messenger RNA level, under the control of various cspA promoters, at 37 and 15°C.

Best Mode For Carrying Out The Invention

The invention relates to expression vectors which lead to the high level expression of subcloned genes in bacterial host cells at low temperatures (i.e., at temperatures less than about 20°C), and which allow the production of proteins in their native and active form.

The invention is based on the premise that the rate of protein folding will be only slightly affected by lowering the temperature to about 15-20°C, while the rate of transcription and protein synthesis, being biochemical reactions, will be greatly slowed down (e.g. 5- to 10-fold). Exploitation of these conditions, as in the context of the present invention, allows individual proteins sufficient time to refold in an independent way, yielding active proteins and preventing the formation of aggregates, without reducing the final yield of the desired protein.

Accordingly, the invention provides vectors which allow for the production of native and active eukaryotic protein in a bacterial host cell.

Fermentation in bacterial host cells containing such vectors is based on a two-stage process. At the first stage there is rapid bacterial growth at high temperatures of about 37-43°C during which only a very low-level of expression of a subcloned gene contained within the vector takes place. At the second stage there is an increase of gene expression of the subcloned gene

upon transfer to the low temperature. Following fermentation, pure, soluble and highly active proteins can be isolated. The increased production of protein that occurs upon transfer of the host cells to low temperature requires utilizing the specifically designed expression systems which are the subject of the present invention. These vectors allow for increased gene expression and production of protein or peptides at a temperature below about 20°C.

By utilizing a high temperature for bacterial growth, the cell carrying the desired gene will grow rapidly without expressing the desired peptide. Following a change to lower temperature in the fermentation system, the expression of the gene encoding the foreign protein will be initiated. Finely tuning the rate of synthesis of the desired protein at low temperature yields high levels of soluble and biologically active proteins. This expression system is of general use and can be used for proteins such as viral proteins, especially those useful for preparing vaccines against viruses, in particular against hepatitis viruses such as hepatitis A, insulin, cytokines such as interleukins such as IL 1-13, in particular IL-2α, interferons, clotting factor genes such as Factors I-XIII, in particular VIII, hormones such as follicle stimulating hormones, any glucocerebrosidase such as β-glucocerebrosidase and other growth factors and, in principle, any desired protein or peptide.

The vectors of the present invention comprise a nucleic acid sequence which comprises a gene encoding a desired protein or peptide, as well as a promoter which is highly active at low temperature. These vectors, upon introduction to a bacterial host cell, enable the high level production of the desired protein or peptide at low temperatures. The protein or peptide is obtained in a

native active form, and therefore no additional
manipulation of the protein, such as protein refolding,
is required.

Accordingly, the present invention provides an
expression vector comprising a nucleic acid sequence
which comprises a gene and a promoter that is capable of
controlling, when the vector is in a bacterial host and
the temperature is lowered to below about 20°C, the
production of a recombinant peptide or protein encoded by
the gene contained within the vector.

In the context of the present invention, a promoter
is a DNA sequence that directs the binding of RNA
polymerase and thereby promotes nascent mRNA synthesis.
The DNA sequences of eukaryotic promoters differ from
those of prokaryotic promoters which means that
eukaryotic signals may not be recognized in prokaryotic
systems, and *vice versa*. Accordingly, the promoters of
the present invention allow transcription of a gene
contained within the vector and, moreover, allow
increased transcription at temperatures below about 20°C.

Generally the gene inserted into the vector will be
foreign (i.e., a gene that is not normally expressed in
the particular host in which the vector will be
introduced), and, preferably, the gene will be of
eukaryotic origin. The gene may be wholly or partially
synthetic. The protein or peptide encoded by the gene
may comprise a full length protein, a polypeptide, or a
chimeric protein.

Also, the vectors of the present invention must be
capable of replication in a bacterial host (i.e., either
autonomously or as part of the host genome), and must be
capable of directing the production of the peptide or
protein in the bacterial host. Thus, the vectors
preferably comprise appropriate control elements (e.g.,

promoters and ribosome binding sites) for commanding the
production of the peptide or protein in the host.

In some cases, the gene contained within the vector
and encoding the protein or peptide will contain the
appropriate regulatory elements such that these elements
need not be present on the vector. In other cases (such
as when a eukaryotic protein is being produced in a
prokaryotic host), it is preferred that the vectors of
the present invention comprise these additional elements.
It is preferred that all of the proper transcription and
translation signal be correctly arranged on the vector,
such that the foreign gene will be properly expressed
within the host, and protein will be translated, when the
temperature is decreased to below at 20°C.

Accordingly, preferably the expression vectors of
the present invention further comprise an initiation
codon (such as an ATG codon) positioned so as to allow
translation of the peptide or protein to initiate from
the initiation codon. Even more preferable is that the
initiation codon be positioned between said promoter and
said gene.

Also, preferably the expression vectors of the
present invention further comprise a ribosome binding
site positioned so as to control translation of said
peptide or protein, preferably positioned between the
promoter and the translation initiation codon.

The presence of these additional control elements on
the vectors may be necessitated by the fact that
translation in prokaryotes, just like transcription, also
does not proceed well in response to eukaryotic signals.
For instance, initiation of translation in prokaryotes
requires the presence of an initiation codon, such as the
ATG codon. If the foreign DNA does not comprise such a
codon, one or more such codons can be provided in the
vectors of the present invention. Preferably the

initiation codon will be placed upstream from the
sequences through which translation is to initiate, and
downstream of the promoters of the present invention
which impart increased transcription at low temperatures
upon subcloned fragments placed under their control.

Similarly, the vectors of the present invention may
also comprise a ribosome binding site (i.e., a "Shine-
Dalgarno sequence"; Shine et al., Nature (1975) 254:34).
It is known that efficient translation in prokaryotes is
dependent on such a sequence, which exhibits
complementarity to the 3' end of the 16S ribosomal RNA
(rRNA), and likely promotes binding of mRNA to ribosomes
by duplexing with the rRNA, thus allowing the correct
positioning of the ribosome on the mRNA. Accordingly, it
is preferable that the ribosome binding site be contained
within the vectors of the present invention upstream of
the translation initiation codon and downstream of the
promoters which impart increased transcription at low
temperatures upon subcloned fragments.

Preferably, the vectors of the present invention
further comprise additional operably-bound DNA sequences
which contain other control elements. For instance
repressor sites involved in the turning off of gene
expression and enhancers involved in the augmentation of
gene expression are contemplated in the context of the
present invention.

The vectors of the present invention comprise a
nucleic acid sequence preferably comprising DNA, which
may be single or double-stranded. The vectors may exist
as plasmids, cosmids (i.e., plasmids comprised of a cos
site) or bacteriophages. In preferred embodiments of the
present invention, the vector comprises a plasmid, or a
bacteriophage, or may comprise a plasmid:bacteriophage
cointegrate which may further be inserted into the
bacterial chromosome.

-12-

Preferred vectors of the present invention wherein the vector comprises a bacteriophage are: λPcspA (λA01131 deposited as ATCC No. 75718) and λA01107 (deposited as ATCC No. 75717). Other preferred vectors include but are not limited to pHG87 and pHG91.

While prokaryotic species such as the bacterium *E. coli* stop growing at about 10°C, the vectors of the present invention allow production at a temperature of less than about 20°C (i.e., at about 15-16°C) of a high level of a peptide or protein encoded by a gene contained within the vector, for example, of the reporter gene lacZ.

The low temperature provides a way to regulate the level of gene expression, allowing the skilled artisan to find a temperature at which the expression rate will be reduced, and providing the maximal time needed for proper protein folding, thus preventing protein aggregation. In other words, as the rate of cell growth is slowed down at lower temperature, the relative time allowed for refolding of individual protein molecules will be increased.

The expression systems described herein are based on two different sets of promoters and regulatory elements. In one embodiment, the expression vectors according to the invention comprise the cold-sensitive pL promoter of phage λ. Descriptions in the literature of the use of the pL promoter of phage λ and its thermosensitive cI repressor mutant have been extensive. Typically, cells are first grown at 30°C until a high cell mass is reached. At that stage, the cells are transferred to 42°C and repression of the pL promoter is lifted, leading to the enhanced expression of a subcloned gene placed under the control of the pL promoter (see, e.g., Giladi et al., J. Mol. Biol. (1992) 227:985-990; Giladi et al., J. Mol. Biol. (1992) 224:937-948; Ptashne, Genetic

-13-

Switch: Phage Lambda and Higher Organisms (Cell Press &
Blackwell Scientific Publications, Cambridge, MA (1992)).

However, the present invention comprises a pL mutant
promoter that is most active at a low temperature (i.e.,
a temperature less than about 20°C). In addition it was
discovered in the context of the present invention that
the wild-type and improved mutants of the pL promoter
(e.g., pL-9G-50) are also highly active at temperatures
less than about 20°C.

A number of pL derivatives were constructed using
conventional recombinant DNA techniques and tested for
promoter response to low temperature as described in
Example 2 below. A 3 base pair mutant at the region from
-4 to +4 of the transcription start site (which altered
the sequence from GCACATCA to GGACAAGA) diminished the
cold-response. Other nucleotide substitutions at other
sites within this region, in particular the sites
appearing in bold text, should provide similar results.
A similar reduction in the cold-response was obtained
when sequence at the -10 region of the pL promoter was
changed from GATACT to TATAAT. Other nucleotide
substitutions at other sites within this region, in
particular the sites appearing in bold text, should
provide similar results. A -9G mutation (in which the
sequence GATACT was changed to GATGCT) resulted in a
significant response upon transfer to low temperature.
Other nucleotide substitutions at other sites within this
region, in particular the site appearing in bold text,
should provide similar results.

pHG309, a pL promoter derivative in which an
internal deletion, spanning nucleotides -40 to -70, was
introduced, retains the response to low temperature but
does not require IHF for full activity. To obtain the
-40 to -78 deletion, two successive PCR reactions were
used. In the first reaction, a pL fragment extending

from -130 to -79 and carrying at the 3' end an additional
12 base pairs from -39 to -28, was engineered using
primers #1815 (5'-TCAGAATTCTCACCTACC-3') and #2319 (5'-
TATGTCAACACCTTTTTTATATG-3'). In the second reaction,
primers #2011 (5'-AAGGATCCAATGCTTCGTTT-3') and the
product of the first PCR reaction were used. This
promoter was found, when present on a multicopy plasmid,
to be about 7 to 15 fold more active than the -9G-50
mutant, depending on growth conditions (as assayed by ß-
galactosidase activity). It is expected that other
mutants which contain lesser deletions in this same area
(so long as the open reading frame is maintained) will
similarly retain low temperature responsiveness and may
also may not require IHF for full activity.

The pL-Plac chimeric promoter was generated in a
similar way, using primer #1815 and a chimeric primer
#4402 (5'-ACGAGCCGGAAGCATAAAGTGTAAATGTTTTTTATATGAA-3') on
a pL template in the first PCR reaction. In the second
reaction, the product of the first reaction was used as
the upstream primer. The universal M13 primer was
employed and the Plac promoter served as template. In
this fusion product, expression is regulated by the lacI
repressor and can be induced by IPTG. The region from -
130 to -79 of pL was fused to Plac (from -36 to +84).
The response of this promoter to low temperature was not
tested.

In another embodiment, the expression vectors of the
invention comprise the cspA promoter. This promoter is
highly responsive to low temperatures, and, in its native
position in the bacterial chromosome, directs the
synthesis of over 13% of the bacterial proteins
synthesized (Tanabe et al., J. Bacteriol. (1992)
174:3867; Goldstein, Proc. Nat. Acad. Sci. (1990)
87:283). The cspA gene is the major cold shock gene that
has been described in bacteria (Goldstein et al., Proc.

Natl. Acad. Sci. (1990) 87:283; Jones et al., J.
Bacteriol. (1992) 174:3903; Tanabe et al., J. Bacteriol.
(1992) 174:3867).

The promoter region of the cspA gene has been
dissected, allowing the identification of a number of
regions involved in the enhancement of transcription.
The strength of this promoter allows certain clones to be
maintained only at high temperatures. The influence of
the cspA promoter on lacZ expression in the context of
the present invention was confirmed as illustrated in the
Examples.

A spontaneous mutation in the -35 region (a T:A to
C:G mutation at -36) greatly increases the partition of
expression between high and low temperature (and see
Figure 19). It is expected that any nucleotide
substitution at this position would provide similar
results. It is further expected that other mutations ± 5
of the -35 site will provide similar results.

Accordingly, preferred vectors of the present
invention comprise a pL promoter or a PcspA promoter.
The pL promoter and PcspA promoter may further comprise a
mutation or mutations. In the context of the present
invention, a mutation results in an alteration of the DNA
sequence, and may consist of an addition, deletion or
substitution in the base sequence. Some mutations of the
PcspA promoter according to the present invention are
contained within, for example, pIK-L and pIK-M.
Similarly, a PcspA mutation according to the present
invention is contained within pL-9G-50.

Other preferred vectors of the present invention
comprise vectors in which the promoter comprises the
sequence of: SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:3; SEQ
ID NO:4; SEQ ID NO:5; or SEQ ID NO:6.

Other preferred vectors include: λA01107 contained
within the cell line deposited with the ATCC and assigned

No. 75717; λPcspA contained within the cell line
deposited with the ATCC and assigned No. 75718; pIK-M
contained within the cell line deposited with the ATCC
and assigned No. 75719; pIK-EL contained within the cell
line deposited with the ATCC and assigned No. 76720; and
the plasmids pIK-L and pIK-P.

The present invention further relates to a host-
vector system for the production of a desired protein in
native active form which comprises an expression vector
according to the present invention in a suitable
bacterial, and preferably *E. coli*, host cell.

Also, the expression of the pL promoter is
positively regulated by HIF, which is a natural component
of *E. coli* host cells. Accordingly, HIF⁺ *E. coli* strains,
which contain the known host integration factor, HIF, are
preferred for use with the vectors of the present
invention comprising the pL promoter.

Generally, even more preferred *E. coli* strains are
those which contain mutations that will increase the
level of protein production and/or stability, or will
correct the folding of the desired gene products. Such
strains are, for example, mutants defective in the lon or
hfl genes, which may improve stability by reducing
proteolysis. Additionally, preferred strains may
comprise mutations in recA or other recombination/repair
genes, such mutations which are known to be involved in
recombination, including deletions, of eukaryotic DNA
contained within bacterial hosts.

Accordingly, the present invention provides a
bacterium containing the vector in which the promoter
comprises the sequence of: SEQ ID NO:1; SEQ ID NO:2; SEQ
ID NO:3; SEQ ID NO:4; SEQ ID NO:5; or SEQ ID NO:6.

Similarly, the present invention comprises a nucleic
acid comprising a vector in which the promoter comprises

the sequence of: SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:3;
SEQ ID NO:4; SEQ ID NO:5; or SEQ ID NO:6.

Other preferred host-vector systems for the
production of ß-gal in native active form comprise the
λPcspA or λA01107 expression vectors in a suitable *E.
coli* host cell, particularly strain A7506.

In a preferred host-vector system, the gene encoding
the desired protein or peptide is integrated into a
suitable *E. coli* host cell chromosome through use of the
phage λ expression vectors. Integration into the
chromosome results in a single copy of the gene fusion
which is extremely stable, and which allows for
performing the fermentation procedure without the use of
antibiotics.

Genes can be stably introduced into the bacterial
chromosome using the method of the present invention.
Under these conditions, the pL promoter can be maintained
in the cell in the absence of the cI repressor. A low
level of expression is found when the cells are grown at
elevated temperatures, and a high level of expression is
found when the cells are transferred to a low
temperature.

In order to further improve the system so as to
allow the use of plasmids and further increase protein
production, a mutant repressor that is active at high
temperature but not at low temperature can be
incorporated within the *E. coli* chromosome or subcloned
into a plasmid vector. When supplied with this mutant
repressor, cell cultures could be grown at 37°C before
being transferred to lower temperature to allow for the
expression of the cloned gene. It is possible to use the
system as plasmids. It is possible that to acquire
greater protein stability, the host has to be grown at
42°C before lowering the temperature. It is probable
that specific growth conditions will have to be

developed, depending on the gene to be expressed. Thus, homologous recombination is not essential.

In contrast to the pL promoter, positive or negative regulators of the cspA promoter have not been identified. Using the appropriate promoter constructs according to the present invention, it is possible to directly manipulate promoter activity. Cells carrying proper fusions will be grown at 37°C or higher temperatures, and fusions will be induced by a change in the culture temperature (i.e., lowering the temperature below about 20°C).

The present invention further provides a method of producing a desired protein encoded by a subcloned gene contained within an expression vector which comprises introducing the expression vector into a suitable host cell, preferably a bacterial host cell, maintaining the cell in culture, and isolating the protein produced.

Cell expansion is facilitated by growing cells between 15 to 45°C. Preferably the cell is maintained in culture at a temperature of about 37°C for a suitable length of time to allow an increase in cell growth, and is then maintained in culture at a temperature below about 20°C (between 10 and 20°C) for a suitable length of time to allow production of protein. Cells are grown at elevated temperature followed by transfer to low temperature, while sampling at four hour intervals (up to 72 hours) in order to determine the highest activity. Samples are also evaluated by protein gel electrophoresis.

Isolation of protein can be by any technique known in the art for the purification of proteins, such as chromatography, centrifugation, differential solubility, isoelectric focusing, etc. However, preferably the foreign protein will be obtained from the bacterial host cell by suitable means whereby the protein is recovered

in native active form. Cells are harvested and lysed
using standard procedures. It is possible that specific
buffer conditions will be required to prevent *in vitro*
aggregation of protein.

Standard culture media, rich or minimal, can be used
in the context of the present invention. As mentioned
above, with suitable vectors, addition of antibiotics to
the culture media can be avoided, which may be of
particular advantage for production of certain proteins.

The following examples further illustrate the
present invention but, of course, should not be
considered as in any way limiting its scope.

EXAMPLE 1: Materials and Methods
Employed in Experiments
Bacterial strains, phages and plasmids

In the present experiments, standard molecular and
genetic techniques such as generation of strains, phages
and plasmids, gel electrophoresis, DNA manipulations
including cloning and sequencing, primer extension
assays, etc., were performed such as are known to those
skilled in the art and are described in detail in
standard laboratory manuals (e.g., Maniatis et al.,
Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold
Spring Harbor, NY, 1992); Ausubel et al., Current
Protocols in Molecular Biology, (1987); Miller, A Short
Course in Bacterial Genetics, (Cold Spring Harbor, NY,
1992)). Restriction enzymes and other enzymes used for
molecular manipulations were purchased from commercial
sources, and used according to recommendations. Strains
were cultured, and phages were maintained, propagated and
titered using standard reagents, media and techniques
(Maniatis et al., Molecular Cloning: A Laboratory Manual,
2nd ed. (Cold Spring Harbor, NY, 1992); Miller,
Experiments in Molecular Genetics (Cold Spring Harbor

-20-

Laboratory Press, Cold Spring Harbor, NY, 1972)).
Preferably, Luria-Bertani (LB) Medium containing Bacto
tryptone (10 g/1), Bacto yeast extract (5 g/1) and NaCl
(10 g/1) was employed for growth of cells.

E. coli strain A7506 is a derivative of CSH50 (ara
Δ(lac-pro) strA thi; described in Miller, Experiments in
Molecular Genetics (Cold Spring Harbor Laboratory Press,
Cold Spring Harbor, NY, 1972)) which carries the λA01107
prophage. Strains A7507, a hip3::cat derivative of
A7506, and A7533, a himA82:Tn10 derivative of A7507, were
generated by Plvir transduction.

Strain A6826 is a CSH50 derivative. Strain CSH50
was found to grow slowly on minimal medium plates
containing the supplements vitamin B_1 and proline. A fast
growing colony of CSH50 was isolated, purified and
designated A6826. Strain 7833 is a λcspA EL-lacZ
derivative of A6826 and strain 7832 is a λcspA S-lacZ
derivative of A6826.

The DNA sequence of the pL promoter region including
the nucleotides flanking the Eco RI and Bam HI sites is
shown in Figure 1, and is set forth in SEQ ID NO:1.
Plasmid pHG87 contains the fragment comprising the wild-
type pL promoter, as shown in Figure 2.

The restriction map of plasmid pHG91 is shown in
Figure 3. Plasmid pHG91 contains the mutant pL promoter
pL-9G-50. The sequence of this mutant promoter is
characterized by an (A:T) to (G:C) mutation at basepair
220, and a GGCC sequence substituting AATT at basepairs
177-180, and is set forth in SEQ ID NO:2.

Plasmid pHG91 was obtained by fusing the pL-9G-50
promoter to the lacZ reporter gene present in plasmid
pHG86 (Giladi et al., J. Mol. Biol. (1992) 224:937-948).
A kanamycin resistance gene (Km^R) was then inserted in the
Pst I restriction site located within the bla gene of
pHG91 to generate plasmid pHG91K. When in full length

-21-

form, the lacZ gene encodes ß-galactosidase (ß-gal) and
the bla gene (sometimes designated amp) encodes a ß-
lactamase and confers host cell resistance to ampicillin.

Phage λA01107 is the product of recombination
between phage λB299 and plasmid pHG91K, as set forth in
Figure 4. Phage λB299 (obtained from R. Weisenberg,
Section on Microbial Genetics, Laboratory of Molecular
Genetics, National Institutes of Health) carries the supF
gene flanked by the truncated lacZ and bla genes.
Recombination between phage λB299 and plasmid pHG91K
results in the replacement of the supF gene by the pL-
lacZ fusion from pHG91K in phage λA01107.

Plasmids phip·, pR87G and pA90D are pEMBL18
derivatives, carrying the wild-type hip gene and its
mutant alleles ßR87G and ßA90D (Menegeritsky et al., J_.
Mol. Biol. (1993) 231:646-657).

The cspA promoter fragment (309 bp in length from
positions -214 to +95; Goldstein et al., PNAS (1990)
87:283-287) was cloned using the polymerase chain
reaction (PCR; PCR Protocols, A Guide to Methods and
Applications, Innis et al., eds., Academic Press, Inc.
(1990)), and was inserted into the plasmid pHG86 upstream
of the reporter lacZ gene. Plasmid pIK86 (shown in
Figure 5) was constructed from pHG86 by inserting Km^R
within the bla gene. A set of constructs containing
different sized portions of the cspA promoter region was
then prepared from pIK86. The different size fragments
carrying the pcspA promoter region were each generated by
PCR using a lambda phage carrying the cspA chromosomal
region. Two primers were also employed: a primer
homologous to the 5' region and carrying an Eco RI
restriction site; and a primer homologous to the 3'
region and carrying a Bam HI restriction site. The
restriction maps of the cspA promoter constructs
generated were determined.

-22-

Of the cspA promoter region-containing plasmids, pIK-EL contains the largest upstream cspA promoter region, as indicated in **Figure 6**. The sequence of the cspA fragment subcloned in the PIK-EL vector is given in **Figure 7** and SEQ ID NO:3. A smaller cspA promoter fragment was subcloned into pIK-L, as indicated in **Figure 8**. The sequence of the cspA fragment subcloned in the pIK-L vector is given in **Figure 9** and SEQ ID NO:4. Similarly, pIK-M contains a still smaller cspA fragment, as indicated in **Figure 10**. The sequence of the cspA fragment subcloned in the pIK-M vector is given in **Figure 11** and SEQ ID NO:5. The smallest cspA promoter fragment was subcloned into pIK-S, as indicated in **Figure 12**. The sequence of the cspA fragment subcloned in the pIK-S vector is given in **Figure 13** and SEQ ID NO:6.

Since cspA is a strong promoter, in order to reduce the level of expression being driven by the scpA promoter at high temperatures, these constructs were subsequently transferred to phage λ by homologous recombination, as set forth in **Figure 14**. The phage was then integrated into an *E. coli* chromosome. All crosses between plasmids and λB299 were carried out in strain A6826.

Preparation of DNA Fragments

DNA fragments used for the primer extension assay were obtained by PCR using Taq Polymerase (Promega Corp. Madison, WI). To obtain the 275-bp pL DNA fragment, primer 1921 5'-AAGAATTCGGGTTTTCTTT-3' (pL positions -228 to -217 as set forth in SEQ ID NO: 7), primer 1526 5'-AAGAGCGTCACCTTC-3' (pL positions +40 to +26 as set forth in SEQ ID NO: 8) and plasmid pHG244 DNA as a template were employed (Giladi et al., <u>J. Mol. Biol.</u>, (1992) <u>224</u>: 937-948). All DNA fragments were end-labeled with (γ^{32}P)ATP (Amersham Corp., Arlington Heights, IL) and purified on a 5% polyacrylamide gel.

-23-

Enzymatic assays

Assays of ß-gal specific activity were carried out according to Miller (Miller, Experiments in Molecular Genetics (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1972)).

EXAMPLE 2: Temperature Response of the pL promoter

To test the influence of temperature on gene expression driven by the attenuated pL-9G-50 promoter, this promoter was fused to the lacZ gene and inserted into the *E. coli* chromosome at the att site using phage λ, and generating strain A7506.

Cultures of strain A7506 were grown in LB medium at various temperatures to saturation, and were assayed for ß-gal activity. As can be seen from **Figure 15**, the levels of ß-gal present in the cell were quite high at low temperatures (i.e., less than about 20°C) and declined precipitously with increases in temperature. This temperature effect was not observed when cultures of strain A7507 were employed. Strain A7507 is a hip derivative of strain A7506 which does not produce host integration factor, a positive regulator of the pL promoter. In strain A7507, levels of ß-gal were low at all temperatures.

These results confirm that gene expression driven by the pL-9G-50 promoter is induced by low temperatures.

EXAMPLE 3:   Temperature Shift Experiments

To study the response of the attenuated pL-9G-50 promoter to temperature changes, temperature shift experiments of the lacZ fusion of this promoter present in the chromosome of strain A7506 were performed.

For these experiments, cultures of strain A7506 (hip⁺) and A7507 (hip⁻) were grown exponentially at 37°C

-24-

or about 15-16°C, and were then transferred to the alternate temperature (i.e., a culture grown at 37°C was transferred to about 15-16°C, and a culture grown at about 15-16°C was transferred to 37°C). Levels of ß-gal as well as cell density were determined at different times after the temperature shift.

When exponentially growing A7506 cells were transferred from 37°C to 15-16°C, a dramatic increase in ß-gal levels was observed as indicated in **Figure 16a**. This increase occurred despite the long lag in cell growth due to the cold shock, as demonstrated in Figure 16b, and was dependent on the presence of the wild-type hip gene, as it was not observed for the A7507 cells under the same conditions.

In contrast, when exponentially growing A7506 cells were transferred from 15-16°C to 37°C, a rapid cessation in ß-gal specific activity was observed, as indicated in Figure 16c. This decrease in ß-gal specific activity correlates with the dilution of the existing enzyme by cell division (as indicated in Figure 16d), possibly because transcription from pL was turned off rapidly after transfer to the higher temperature.

These results confirm that the attenuated pL-9G-50 promoter is able to drive a high level of gene expression upon transfer from 37°C to about 15-16°C.

EXAMPLE 4: Effect of Temperature on Promoter Activity

The pL-9G-50 and PcspA promoters were each fused to the lacZ gene in an operon fusion, transferred to phage λB299 by homologous recombination, and inserted at the att site of the bacterial chromosome as described above. Strain N99 was employed as a control to measure plac activity from its authentic operon (Ueshima et al., Mol. Gen. Genet. (1989) 215:185-189). These experiments were

-25-

done to determine whether the temperature response of the pL promoter is unique to that promoter.

For these experiments, cell cultures were grown to saturation in LB medium at 37°C or about 15-16°C, and were then assayed for ß-gal activity. Results are as summarized in Table 1.

TABLE 1

	ß-gal units	
Promoter	15-16°C	37°C
pL-9G-50	3860	260
plac	5950	7320
PcspA	1430	85

These results confirm that the PcspA promoters as well as the pL-9G-50 promoter are able to drive a high level of gene expression at low temperatures (i.e., less than about 20°C). Expression driven by these promoters at about 15-16°C approaches that driven by the strong promoter plac. In contrast, expression of plac is reduced about 10-20% at 15-16°C as compared with at 37°C.

These results suggest that the higher enzyme levels observed for the pL promoter at low temperatures are due to increased pL transcription rather than to increased stability of the enzyme, or to more efficient protein translation. Contrary to the plac promoter, the cspA promoter was 20-fold more active at 15-16°C than at 37°C. It is noteworthy that the pL promoter showed the same temperature activation profile as PcspA. This temperature response is an intrinsic property of the pL promoter, and individual promoters appear to have an inherent response to temperature which is embedded within the promoter DNA sequence. The extent of response of the

pL promoter to low temperatures, i.e., 14-fold higher at 15-16°C than at 37°C, is similar to that of the cspA promoter, yet these two promoters do not share DNA sequence similarities that indicate why they are more active at low temperature.

Thus, the pL-9G-50 and PcspA promoters minimally drive gene expression at temperatures at which bacterial cell growth and replication is optimized (i.e., at about 37°C). This validates that the promoters can be employed to obtain maximal gene expression at temperatures at which bacterial cell growth and replication are minimized (i.e., less than about 20°C).

EXAMPLE 5: Single Copy Chromosomal Fusion by Recombination between Phage λ and Plasmid

Similar experiments as in Example 4 done with the various pIK-cspA constructs confirm that these also are strong promoters, and that in fact, the EL and L constructs are difficult to maintain in host cells, possibly due to lethality as a consequence of a high level of gene expression. In order to reduce the level of expression driven by the cspA constructs at high temperatures, the constructs were transferred to phage λB299 by homologous recombination (as indicated in Figure 14A-B). The resultant phages were then integrated into the bacterial chromosome, resulting in a single copy per cell.

As can be seen from Table 2, gene expression driven by the various cspA promoters as reflected in β-gal levels is temperature-regulated and is rather high in the resultant strains even though these strains contain only a single copy of the lacZ gene fusions.

-27-

TABLE 2

Single Copy	β-gal Units	
	about 20°C	37°C
λIK-EL	20000	1000
λIK-M	24000	2000
λIK-S	9000	900
λIK-P	11170	2440

The single copy system is extremely stable and allows for performing the fermentation procedure without the use of antibiotics. Similar results to those as shown above for the pL promoter demonstrate that the pL wild-type promoter, which cannot be maintained in the absence of repressor, can direct the expression of lacZ in a single copy.

These results confirm that the various cspA promoters (as well as the pL-9G-50 promoter) can effectively drive gene expression at low temperatures resulting in a high level of translated protein when present as only a single copy integrated into the bacterial chromosome.

Example 6: Influence of PcspA promoter on lacZ expression at low temperatures

To test the PcspA promoter influence on lacZ expression, cells carrying the EL-lacZ fusion (i.e., strain A7833), the M-lacZ fusion (i.e., strain A7831) and the S-lacZ fusion (i.e., strain A7832) on phage λ as a single copy in the bacterial chromosome, were examined.

For these experiments, cultures were grown in LB at 37°C, and were transferred to about 15-16°C at time 0. Samples were taken at 1, 2, 4 and 6 hours intervals, and RNA was isolated and assayed by primer extension using

labeled primers and reverse transcriptase. Radioactivity of the band corresponding to the transcription start site was determined.

As can be seen in Figure 17, the endogenous cspA mRNA (i.e., □) is present at high levels only one hour after transfer to the low temperature. In contrast, EL-lacZ (i.e., ●) and M-lacZ (i.e., ■) mRNAs are maintained at high levels for longer time periods. Only basal levels of S-lacZ (i.e., ▲) mRNA were observed at all temperatures.

These results confirm that the EL and M promoters may be used to drive gene expression at low temperatures, and that the increased expression from these promoters is reflected in an increased level of transcribed mRNA.

All of the references cited herein are hereby incorporated in their entireties by reference.

While this invention has been described with an emphasis upon a preferred embodiment, it will be obvious to those of ordinary skill in the art that variations in the preferred methods may be used, including variations due to improvements in the art, and that it is intended that the invention be practiced otherwise than as specifically described herein, to encompass these variations. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

SEQUENCE LISTING

(1)   GENERAL INFORMATION:

    (i)   APPLICANT: Oppenheim, Amos B.
                        Giladi, Hilla
                        Goldenberg, Daniel
                        Kobi, Simi
                        Azar, Idit

    (ii) TITLE OF INVENTION: VECTORS AND TRANSFORMED HOST CELLS
           FOR RECOMBINANT PROTEIN PRODUCTION AT REDUCED
           TEMPERATURES

    (iii) NUMBER OF SEQUENCES: 8

    (iv) CORRESPONDENCE ADDRESS:
        (A)   ADDRESSEE: Leydig, Voit & Mayer, Ltd.
        (B)   STREET: 700 Thirteenth Street, N.W., Suite 300
        (C)   CITY: Washington
        (D)   STATE: D.C.
        (E)   COUNTRY: US
        (F)   ZIP: 20005

    (v)   COMPUTER READABLE FORM:
        (A)   MEDIUM TYPE: Floppy-disk
        (B)   COMPUTER: IBM PC compatible
        (C)   OPERATING SYSTEM: PC-DOS/MS-DOS
        (D)   SOFTWARE: PatentIn Release #1.0, version #1.25

    (vi) CURRENT APPLICATION DATA:
        (A)   APPLICATION NUMBER: US
        (B)   FILING DATE: July 21, 1994
        (C)   CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
        (A)   NAME: Rose, Herbert C.
        (B)   REGISTRATION NUMBER: 28946
        (C)   REFERENCE/DOCKET NUMBER: 60170

    (ix) TELECOMMUNICATION INFORMATION:
        (A)   TELEPHONE: (202) 737-6770
        (B)   TELEFAX: (202) 737-6776

(2)   INFORMATION FOR SEQ ID NO:1:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 343 base pairs

   (B) TYPE: nucleic acid

   (C) STRANDEDNESS: double

   (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CGGGTTTTCT TTGCCTCACG ATCGCCCCCA AAACACATAA ATAAATAGAT ACAACTCACT  60
AAACATAGCA ATTCAGATCT CCCCTGCAAA AAATAAATTC ATATAAAAAA CATACAGATA 120
ATCTCTGGCG GTGTTGACAT AAATACCACT GGCGGTGATA CACTGACCAC CATGAAGGTG 180
ACGCTCTTAA AAATTAAGCC CCAATTGTAT TTATTGAAAA CTCACCTACC AAACAATGCC 240
ACCATCTGCG GTGATAAATT CTGAGCACAT CAGCAGGACG CTGAAGAAGG GCAGCATTCA 300
AAGCAGAAGG CTTTGGGGTG TGTGATACGA AACGAAGCAT TGG                   343
```

(3) INFORMATION FOR SEQ ID NO:2:

  (i) SEQUENCE CHARACTERISTICS:

   (A) LENGTH: 343 base pairs

   (B) TYPE: nucleic acid

   (C) STRANDEDNESS: double

   (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
CGGGTTTTCT TTGCCTCACG ATCGCCCCCA AAACACATAA CCAATTGTAT TTATTGAAAA  60
ATAAATAGAT ACAACTCACT AAACATAGCA ATTCAGATCT CTCACCTACC AAACAATGCC 120
CCCCTGCAAA AAATAAATTC ATATAAAAAA CATACAGAT ACCATCTGCG  GTGATAGGCC 180
ATCTCTGGCG GTGTTGACAT AAATACCACT GGCGGTGATG CTGAGCACAT CAGCAGGACG 240
CACTGACCAC CATGAAGGTG ACGCTCTTAA AAATTAAGCC CTGAAGAAGG GCAGCATTCA 300
AAGCAGAAGG CTTTGGGGTG TGTGATACGA AACGAAGCAT TGG                   343
```

(4) INFORMATION FOR SEQ ID NO:3:

  (i) SEQUENCE CHARACTERISTICS:

   (A) LENGTH: 280 base pairs

   (B) TYPE: nucleic acid

   (C) STRANDEDNESS:

   (D) TOPOLOGY:

  (ii) MOLECULE TYPE: DNA (genomic)

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
CAGACGCGTG AAGCCTTCAA GTGCCGAACT AAAATTGATG CGTTTGATTC AAGCCAACCC  60
GGCATTAAGT AAGCAGTTGA TGGAATAGAC TTTTATCCAC TTTATTGCTG TTTACGGTCC 120
TGATGACAGG ACCGTTTTCC AACCGATTAA TCATAAATAT GAAAAATAAT TGTTGCATCA 180
CCCGCCAATG CGTGGCTTAA TGCACATCAA CGGTTTGACG TACAGACCAT TAAAGCAGTG 240
TAGTAAGGCA AGTCCCTTCA AGAGTTATCG TTGATACCCC TCGTAGTGCA            290
```

(5) INFORMATION FOR SEQ ID NO:4:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 185 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS:
        (D)   TOPOLOGY:
    (ii)   MOLECULE TYPE: DNA (genomic)
    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:4:
TGCTGTTTAC GGTCCTGATG ACAGGACCGT TTTCCAACCG ATTAATCATA AATATGAAAA 60
ATAATTGTTG CATCACCCGC CAATGCGTGG CTTAATGCAC ATCAACGGTT TGACGTACAG 120
ACCATTAAAG CAGTGTAGTA AGGCAAGTCC CTTCAAGAGT TATCGTTGAT ACCCCTCGTA 180
GTGCA   185

(6)   INFORMATION FOR SEQ ID NO:5:
    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 140 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS:
        (D)   TOPOLOGY:
    (ii)   MOLECULE TYPE: DNA (genomic)
    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:5:
TCATAAATAT GAAAAATAAT TGTTGCATCA CCCGCCAATG CGTGGCTTAA TGCACATCAA 60
CGGTTTGACG TACAGACCAT TAAAGCAGTG TAGTAAGGCA AGTCCCTTCA AGAGTTATCG 120
TTGATACCCC TCGTAGTGCA   140

(7)   INFORMATION FOR SEQ ID NO:6:
    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 121 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS:
        (D)   TOPOLOGY:
    (ii)   MOLECLTLE TYPE: DNA (genomic)
    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:6:
TTGTTGCATC ACCCGCCAAT GCGTGGCTTA ATGCACATCA ACGGTTTGAC GTACAGACCA 60
TTAAAGCAGT GTAGTAAGGC AAGTCCCTTC AAGAGTTATC GTTGATACCC CTCGTAGTGC 120
A   121

(8)   INFORMATION FOR SEQ ID NO:7:
    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 19 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (synthetic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAGAATTCGG GTTTTCTTT   19

(9)   INFORMATION FOR SEQ ID NO:8:

    (i)   SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 15 base pairs

        (B)   TYPE: nucleic acid

        (C)   STRANDEDNESS: single

        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (synthetic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAGAGCGTCA CCTTC   15

Claims

1. An expression vector comprising a nucleic acid sequence which comprises a gene and a promoter that is capable of controlling, when the vector is in a bacterial host and the temperature is lowered to below about 20°C, the production of a peptide or protein encoded by said gene.

2. The vector of claim 1 wherein said nucleic acid sequence further comprises an initiation codon positioned between said promoter and said gene so as to allow translation of said peptide or protein to initiate at said initiation codon.

3. The vector of claim 2 wherein said nucleic acid sequence further comprises a ribosome binding site positioned so as to control translation of said peptide or protein.

4. The vector of claim 3 wherein said ribosome binding site is positioned between said promoter and said initiation codon.

5. The vector of claim 4 wherein said nucleic acid sequence further comprises additional operably-bound DNA sequences which contain other control elements.

6. The vector of claim 1 wherein said vector is circular and comprises double-stranded DNA.

7. The vector of claim 1 wherein said vector comprises a bacteriophage.

8. The vector of claim 1 wherein said promoter is selected from the group consisting of the pL and PcspA promoters.

9. The vector of claim 8 wherein said promoter is a pL promoter.

10. The vector of claim 8 wherein said promoter is a PcspA promoter.

11. The vector of claim 9 wherein said pL promoter comprises a mutation or mutations.

12. The vector of claim 1 wherein said promoter comprises the sequence of SEQ ID NO:1.

13. The vector of claim 1 wherein said promoter comprises the sequence of SEQ ID NO:2.

14. The vector of claim 1 wherein said promoter comprises the sequence of SEQ ID NO:3.

15. The vector of claim 1 wherein said promoter comprises the sequence of SEQ ID NO:4.

16. The vector of claim 1 wherein said promoter comprises the sequence of SEQ ID NO:5.

17. The vector of claim 1 wherein said promoter comprises the sequence of SEQ ID NO:6.

18. The vector of claim 1 which comprises λA01107 contained   within the cell line of ATCC No. 75717.

19. The vector of claim 1 which comprises λPcspA contained within the cell line of ATCC No. 75718.

20. The vector of claim 1 which comprises plasmid pIK-M contained within the cell line of ATCC No. 75719.

21. The vector of claim 1 which comprises plasmid pIK-EL contained within the cell line of ATCC No. 76720.

22. A host cell containing the vector of claim 12.

23. A host cell containing the vector of claim 13.

24. A host cell containing the vector of claim 14.

25. A host cell containing the vector of claim 15.

26. A host cell containing the vector of claim 16.

27. A host cell containing the vector of claim 17.

28. A nucleic acid comprising the vector of claim 12.

29. A nucleic acid comprising the vector of claim 13.

30. A nucleic acid comprising the vector of claim 14.

31. A nucleic acid comprising the vector of claim 15.

32. A nucleic acid comprising the vector of claim 16.

33.   A nucleic acid comprising the vector of claim
17.

34.   A method of producing a protein, which method
comprises introducing the expression vector of claim 1
into a suitable host cell, maintaining said cell in
culture so as to produce a protein, and isolating said
protein.

35.   The method of claim 34 wherein said cell is
maintained in culture at a temperature of about 37°C for
a suitable length of time to allow an increase in cell
growth, and is then maintained in culture at a
temperature below about 20°C for a suitable length of
time to allow production of said protein.

36.   The vector of claim 8 wherein said promoter is
a pL promoter in which the -4 to +4 region shown in
Figure 1 has been mutated from GCACATCA to GGACAAGA.

37.   The vector of claim 8 wherein said promoter is
a pL promoter in which the -10 region shown in Figure 1
has been mutated from CATACT to TATAAT.

38.   The vector of claim 8 wherein said promoter is
a pL promoter in which the -40 to -78 region shown in
Figure 1 has been deleted.

39.   The vector of claim 8 wherein said promoter is
a pL promoter in which the -9 region shown in Figure 1
has been mutated from CATACT to GATGCT.

pL DNA

EcoRI
GGCCTCAGGCGCCGGGGTTTCTTTGCCTCACGATCGCCCCAAAACACATAACCATTGTATTTATTGAAAAA

CCGGAGTCGCGGCCCAAAAGAAACGGAGTGCTAGCGGGGTTTGTGTATTGGTTAACATAAATAACTTTTT

L2

TAAATAGATACAACTTCACTAAACATAGCAATTCAGATCTCTCACCTACCAAAACAATGCCCCCTGCAAAAA

ATTTATCTATGTTGAGTGATTTGTATCGTTAAGTCTAGAGAGTGGATGGGTTTGTTACGGGGGACGTTTTT

L1   BglII   (-50)   pL2   (sex1)
                          GGCC   T
TAAATTCATATATAAAAAACATACAGAT|GACCATCTGGCGGTGATAAATTATCTCTGGCGGTGTTTGACATAAAATA|
            -35   -10   -35

FIG. 1A

RECTIFIED SHEET (RULE 91)

ATTAAGTATATTTTTGTATGTCTAT TGGTAGAGGCGCCACTATTTAATAGAGACCGCCACAACTGTATTTAT

OL3 OL2 HincII

(-9G)
G
CCACTGGCGGGTGATACTGAGCACACATCAGCAGGACGCACCACCATGACCATGAAGGTGACGCTCTTAAAAATTAA
-10 pL1 +1

OL1
GGTGACCGCCACTATGACTCGTGTAGTCGTCCTGCGTGGTACTTCCACTGCGAGAATTTTTAATT

nutL
GCCCTGAAGAGGGCAGCATTCAAAGCAGAAGGCTTTGGGGTGTGTGATACGAAACGAAGCATTGGCC

CGGGACTTCTTCCCGTCGTAAGTTTCGTCTTCCGAAACCCCACACTATGCTTTGCTTCGTAACCGG
BamHI

FIG. 1B

FIG.5

pIK86
7.65 Kb

EcoRI
SmaI
BamHI
trp
T1₄
'bla
pstI
KmR
bla'
PstI
bla'
lacZ
lacY'
ori

FIG.3

pHG91
6.65 Kb

EcoRI
BamHI
PL-96-50
T1x4
PstI
bla
lacZ
lacY'
ori

FIG.2

pHG87
6.65 Kb

EcoRI
BamHI
PL wt
T1x4
PstI
bla
lacZ
lacY'
ori

FIG. 4A

FIG. 4B

FIG.10

pIK-M
7.80 Kb

EcoRI · BamHI · pcspA · T1₄ · 'bla · pstI · KmR · bla' · PstI · ori · lacY' · lacZ

FIG.8

pIK-L
7.86 Kb

EcoRI · BamHI · pcspA · T1₄ · 'bla · pstI · KmR · bla' · PstI · ori · lacY' · lacZ

FIG.6

pIK-EL
7.96 Kb

EcoRI · BamHI · pcspA · T1₄ · 'bla · pstI · KmR · bla' · PstI · ori · lacY' · lacZ

181 GACAGGATTAAAAATCGATGATTTCGCCCGGGTTTGGGCGTATCAGTCGCCATGGTAAA 240
    CTGTCCTAATTTTAGCTACTAAAGCGGGCCCAAAACCCGCATAGTCAGCGGTACCATTT

        EL
        →
241 GGAATGGGAATCCAGACGCGTGAAGCCTTCAAGTGCCGAACTAAAATTGATGCGTTTGAT 300
    CCTTACCCTTAGGTCTGCGCACTTCGGAAGTTCACGGCTTGATTTTAACTACGCAAACTA

301 TCAAGCCAACCCGGCATTAAGTAAGCAGTTGATGGAATAGACTTTATCCACTTTATTGC 360
    AGTTCGGTTGGGCCGTAATTCATTCGTCAACTACCTTATCTGAAATAGGTGAAATAACG

361 TGTTTACGGTCCTGATGACAGGACCGTTTTCCAACCGATTAATCATAAATATGAAAAATA 420
    ACAAATGCCAGGACTACTGTCCTGGCAAAAGGTTGGCTAATTAGTATTTATACTTTTTAT

FIG. 7A

PcspA

—35
—10
+1

```
      -35                          -10          +1
421  ATTGTTGCATCACCCGCCAATGCGTGGCTTAATGCACATCAACGGTTTGACGTACAGACC  480
     TAACAACGTAGTGGGCGGTTACGCACCGAATTACGTGTAGTTGCCAAACTGCATGTCTGG

481  ATTAAAGCAGTGTAGTAAGGCAAGTCCCTTCAAGAGTTATCGTTGATACCCCTCGTAGTG  540
     TAATTTCGTCACATCATTCCGTTCAGGGAAGTTCTCAATAGCAACTATGGGGAGCATCAC

541  CACATTCCTTTAACGCTTCAAAATCTGTAAAGCACGCCATATCGCCGAAAGGCACACTTA  600
     GTGTAAGGAAATTGCCGAAGTTTAGACATTTCGTGCGGTATAGCGGCTTTCCGTGTGAAT
```

EL

FIG. 7B

```
                                                  240                                    300                          L   →              360
181  GACAGGATTAAAAATCGATGATTTCGCCCGGGTTTTGGGCGTATCAGTCGCCATGGTAAA
     CTGTCCTAATTTTTAGCTACTAAAGCGGGCCCAAAACCGCATAGTCCCGGTACCATTT

241  GGAATGGGAATCCAGACGCGTGAAGCCTTCAAGTGCCGAACTAAAAATTGATGCGTTTGAT
     CCTTACCCTTAGGTCTGCGCACTTCGGAAGTTCACGGCTTGATTTTAACTACGCAAACTA

301  TCAAGCCAACCCGGCATTAAGTAAGCAGTTGATGGAATAGACTTTTATCCACTTTATTGC
     AGTTCGGTTGGGCCGTAATTCATTCGTCAACTACCTTATCTGAAAATAGGTGAAATAACG
```
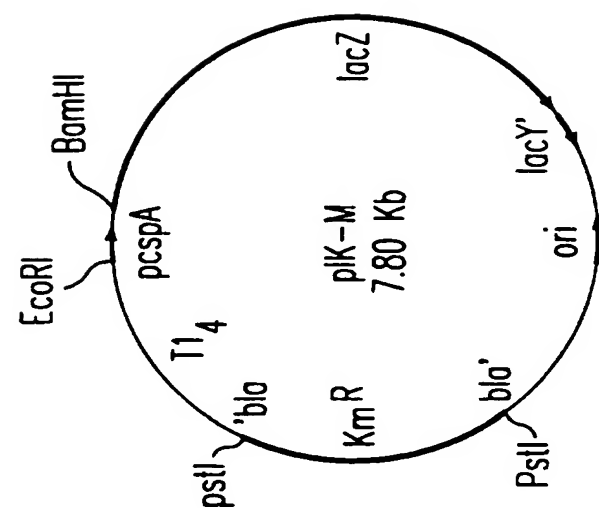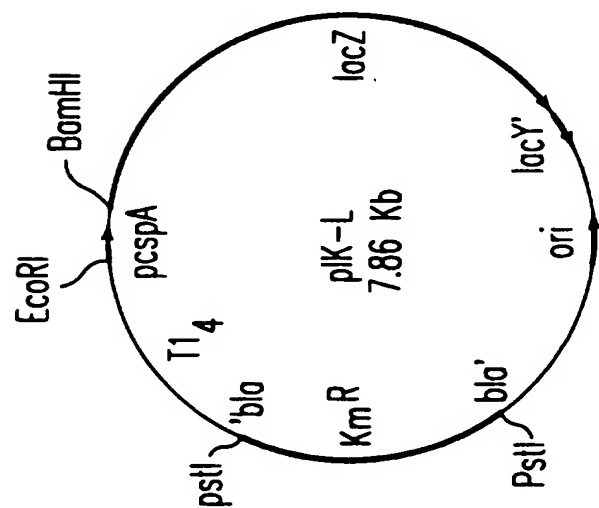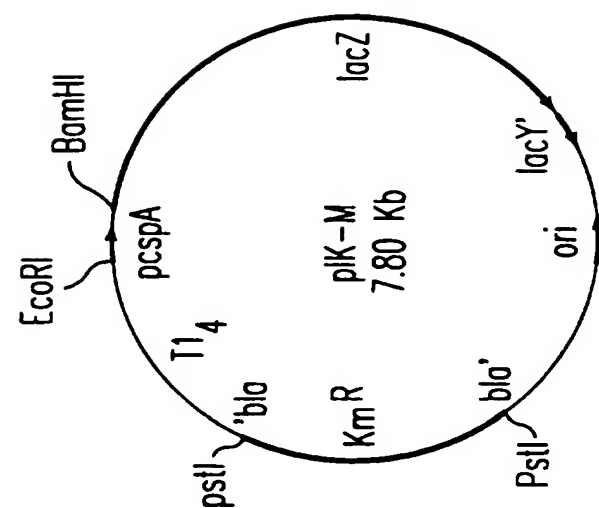
FIG. 9A

```
361  TGTTTACGGTCCTGATGACAGGACCGTTTCCAACCGATTAATCATAAATATGAAAAATA  420
     ACAAATGCCAGGACTACTGTCCTGGCAAAAGGTTGGCTAATTAGTATTTATACTTTTTAT

                                                      +1
                                                      ┌→
     ──────                  ──────
       -35                    -10
421  ATTGTTGCATCACCCGCCAATGCGGTGGCTTAATGCACATCAACGGTTTGACGTACAGACC  480
     TAACAACGTAGTGGGCGGTTACGCACCGAATTACGTGTAGTTGCCAAACTGCATGTCTGG

481  ATTAAAGCAGTGTAGTAAGGCAAGTCCCTTCAAGAGTTATCGTTGATACCCCTCGTAGTG  540
     TAATTTCGTCACATCATTCCGTTCAGGGAAGTTCTCAATAGCAACTATGGGAGCATCAC

541  CACATTCCTTTAACGCTTCAAAATCTGTAAAGCACGCCATATCGCCGAAAGGCACACTTA  600
     GTGTAAGGAAATTGCGAAGTTTTAGACATTCGTGCGGTATAGCGGCTTTCCGTGTGAAT
                  ←┘
                   └
```
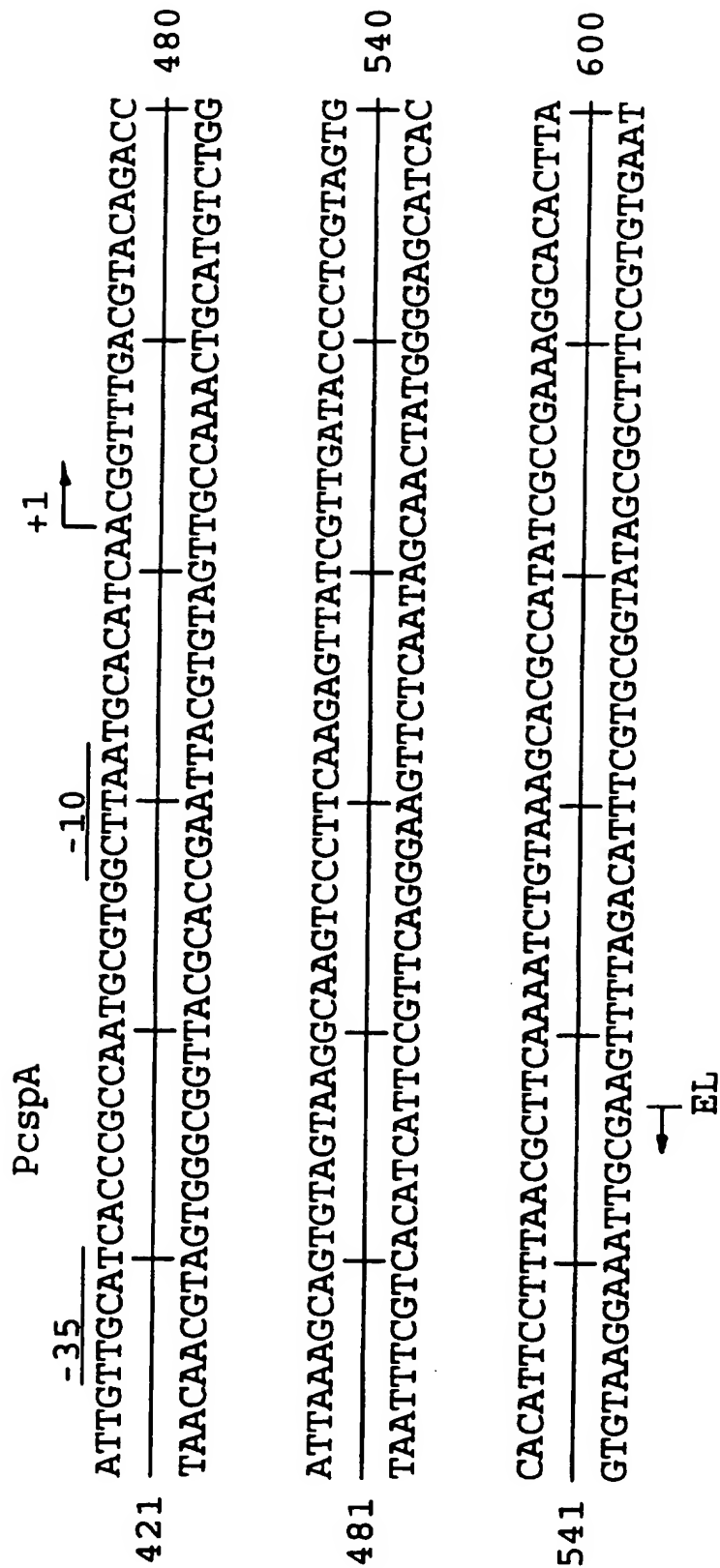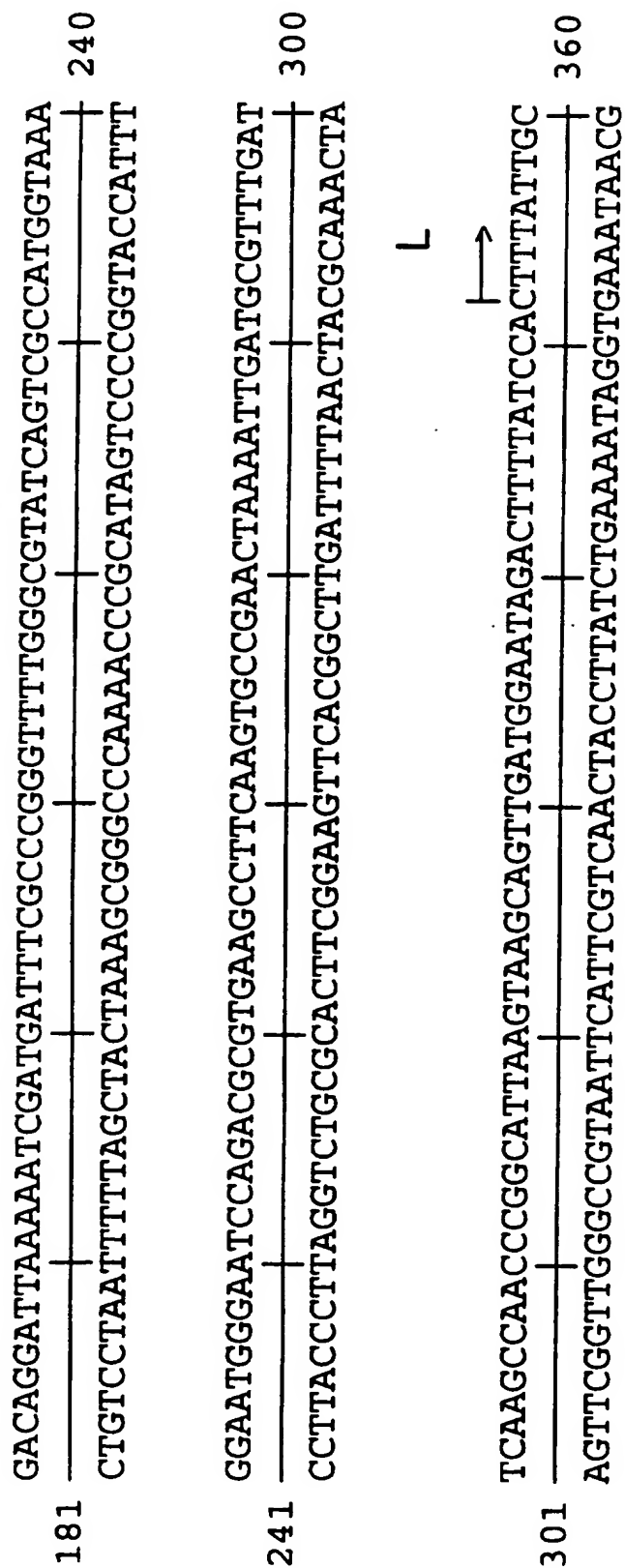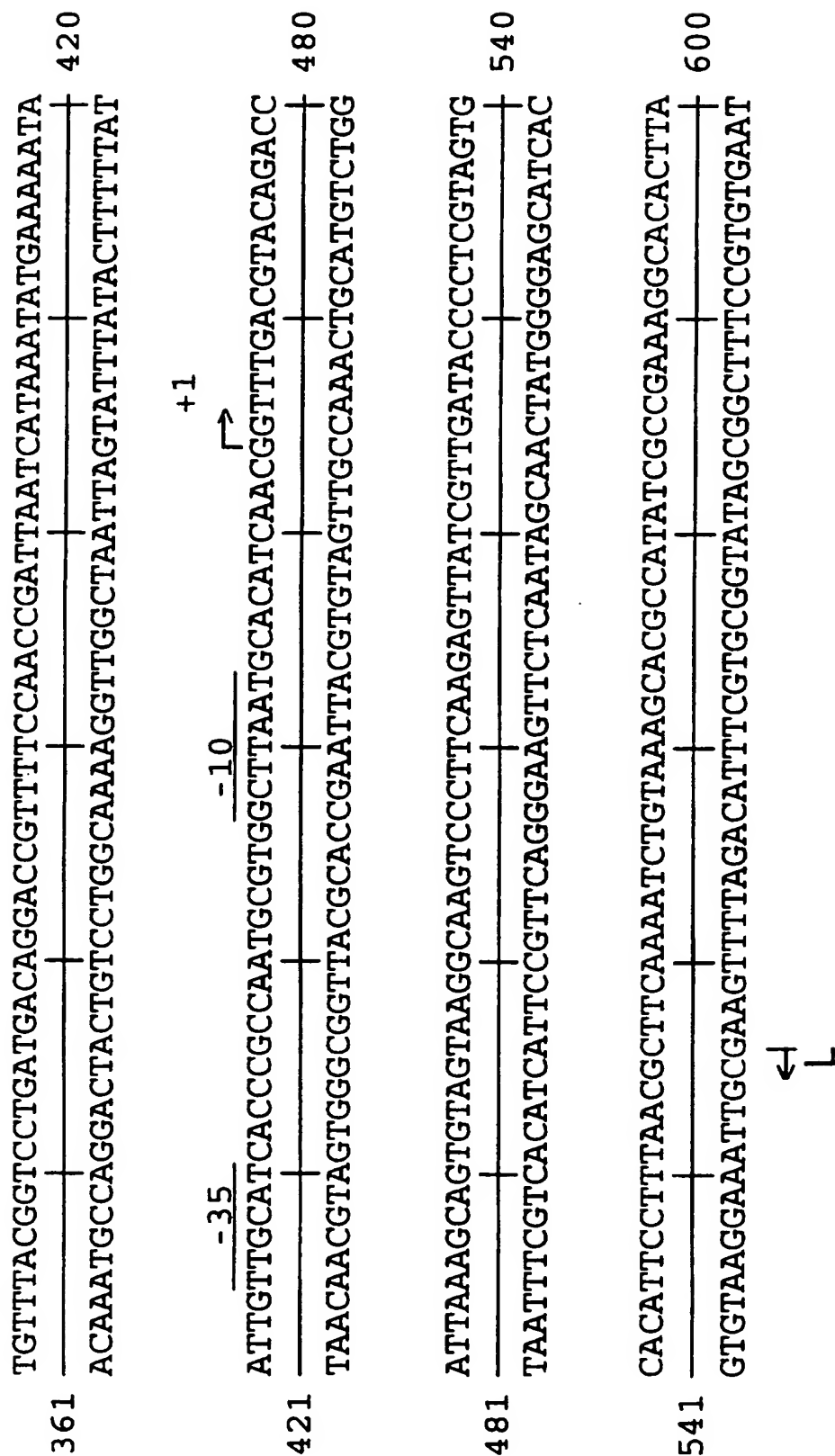
FIG.9B

181 GACAGGATTAAAAATCGATGATTTCGCCCGGGTTTGGGCGTATCAGTCGCCATGGTAAA 240
    CTGTCCTAATTTTTAGCTACTAAAGCGGGCCCAAAACCCGCATAGTCAGCGGTACCATTT

241 GGAATGGGAATCCAGACGCCGTGAAGCCTTCAAGTGCCGAACTAAAATTGATGCGTTTGAT 300
    CCTTACCCTTAGGTCTGCGGCACTTCGGAAGTTCACGGCTTGATTTTAACTACGCAAACTA

301 TCAAGCCAACCCGGCCATTAAGTAAGCAGTTGATGGAATAGACTTTTATCCACTTTATTGC 360
    AGTTCGGTTGGGCCGGTAATTCATTCGTCAACTACCTTATCTGAAAATAGGTGAAATAACG

M
↦

361 TGTTTACGGTCCTGATGACAGGACCGTTTTCCAACCGATTAATCATAAATATGAAAAATA 420
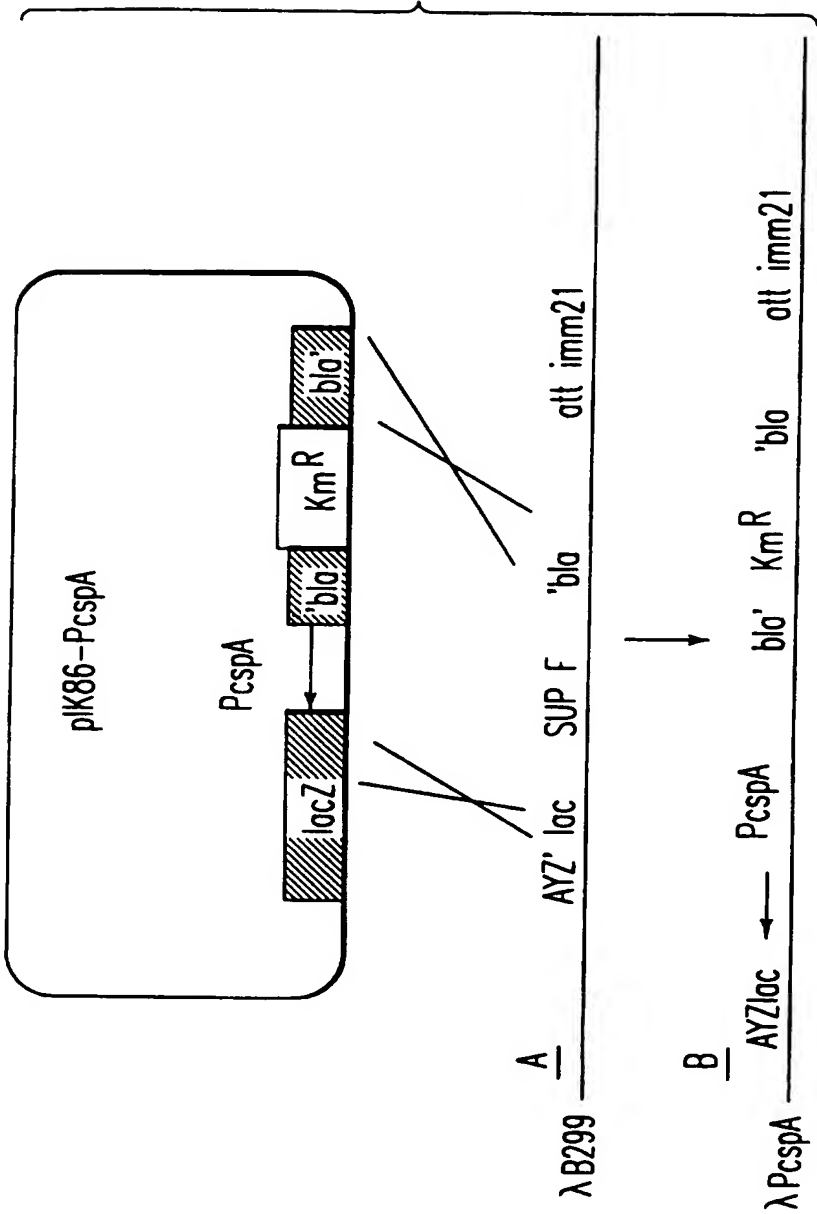   ACAAATGCCAGGACTACTGTCCTGGCAAAAGGTTGGCTAATTAGTATTTATACTTTTTAT

FIG. 11A

-35

-10   +1

421 ATTGTTGCATCACCCGCCAATGCCGTGGCTTAATGCACATCAACGGTTTGACGTACAGACC 480
    TAACAACGTAGTGGGCGGTTACGCACCGAATTACGTGTAGTTGCCAAACTGCATGTCTGG

481 ATTAAAGCAGTGTAGTAAGGCAAGTCCCTTCAAGAGTTATCGTTGATACCCCTCGTAGTG 540
    TAATTTCGTCACATCATTCCGTTCAGGGAAGTTCTCAATAGCAACTATGGGGAGCATCAC

541 CACATTCCTTTAACGCTTCAAAATCTGTAAAGCACGCCCATATCGCCGAAAGGCACACTTA 600
    GTGTAAGGAAATTGCGAAGTTTTAGACATTTCGTGCCGTATAGCGGCTTTCCGGTGTGAAT

M

FIG. 11B

FIG. 14

FIG. 12

181 GACAGGGATTAAAAATGATGATTTCGCCCGGGTTTGGGCGTATCAGTCGCCATGGTAAA 240
    CTGTCCTAATTTTTAGCTACTAAAGCGGGCCCAAACCCGCATAGTCAGCGGTACCATTT

241 GGAATGGGGAATCCAGACGCGGTGAAGCCTTCAAGTGCCGAACTAAAATTGATGCGTTTGAT 300
    CCTTACCCCTTAGGTCTGCGCACTTCGGAAGTTCACGGCTTGATTTTAACTACGCAAACTA

301 TCAAGCCAACCCGGGCATTAAGTAAGCAGTTGATGGAATAGACTTTTATCCACTTTATTGC 360
    AGTTCGGTTGGGCCGTAATTCATTCGTCAACTACCTTATCTGAAAATAGGTGAAATAACG

361 TGTTTACGGTCCTGATGACAGGACCGTTTCCAACCGATTAATCATAAATATGAAAAATA 420
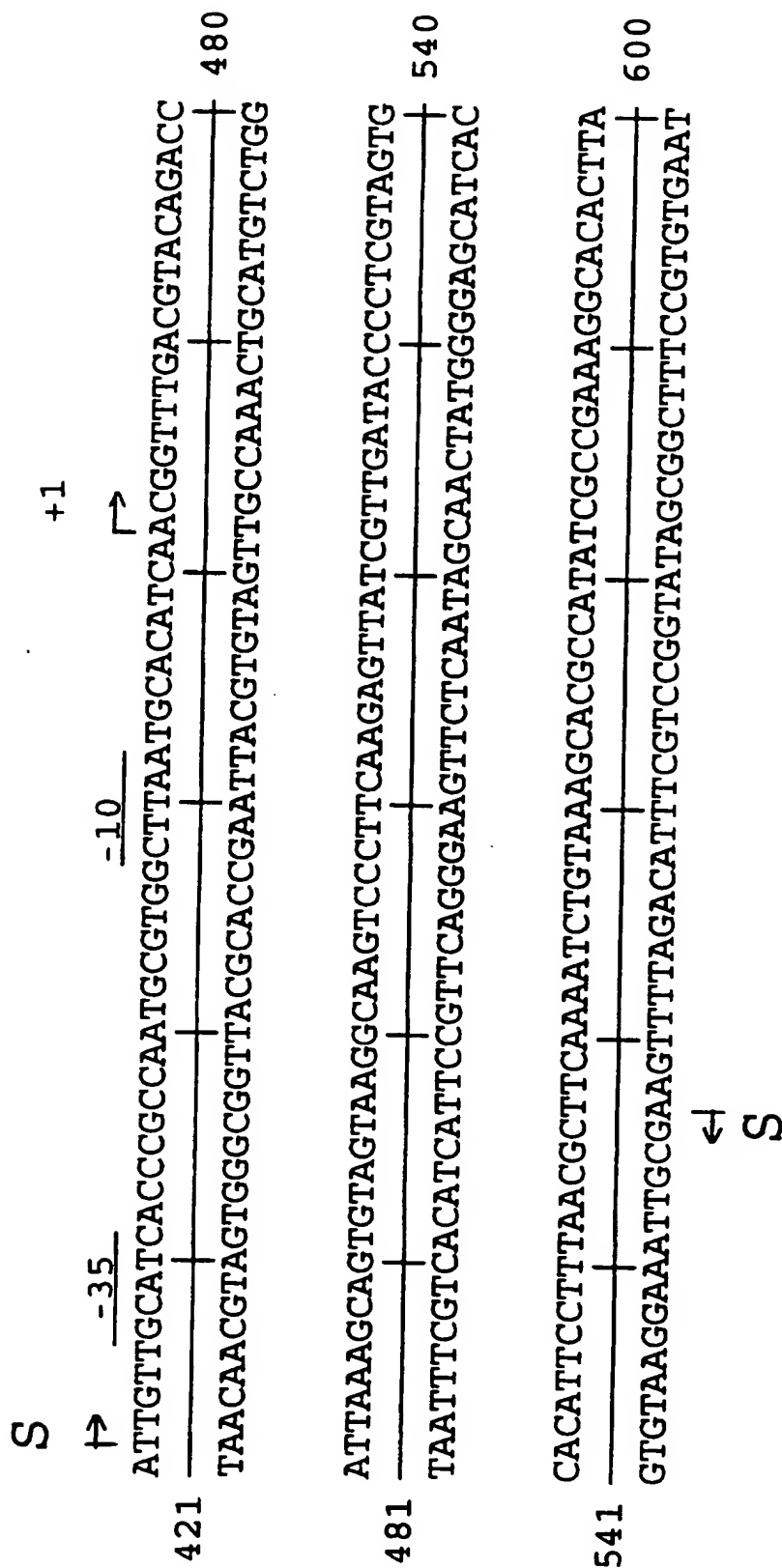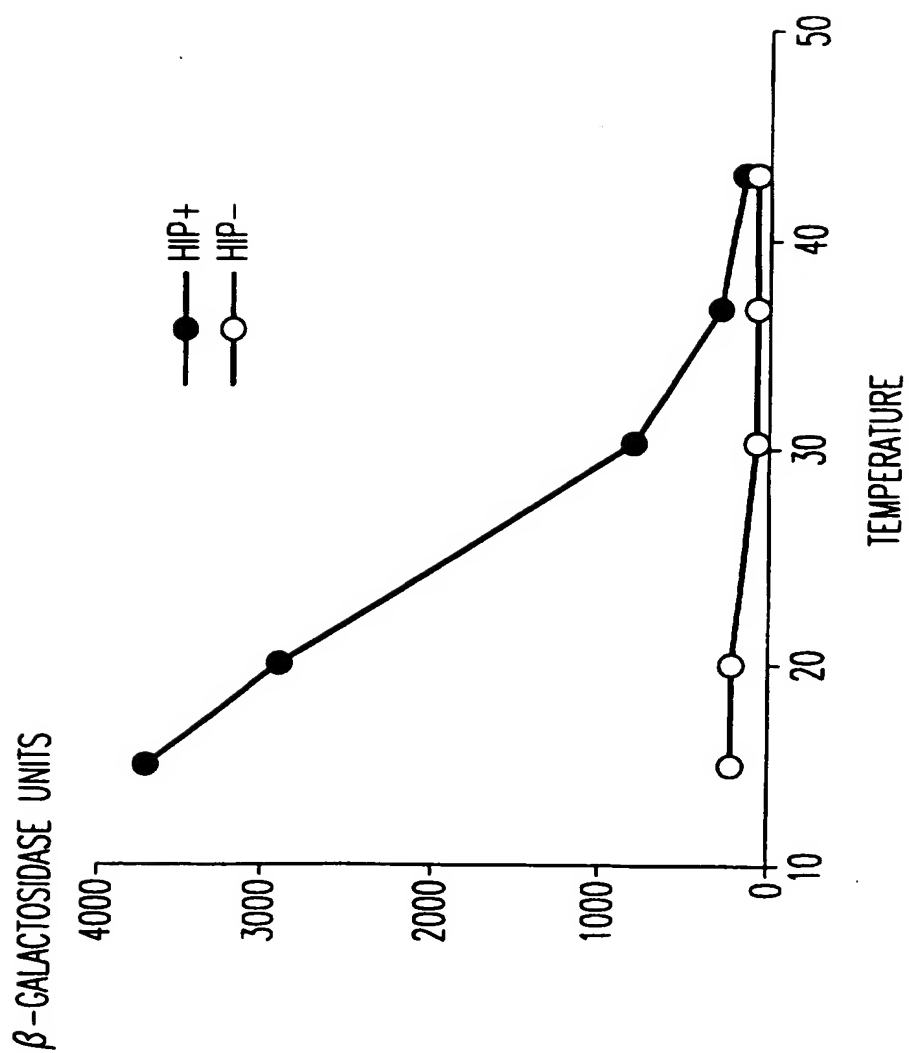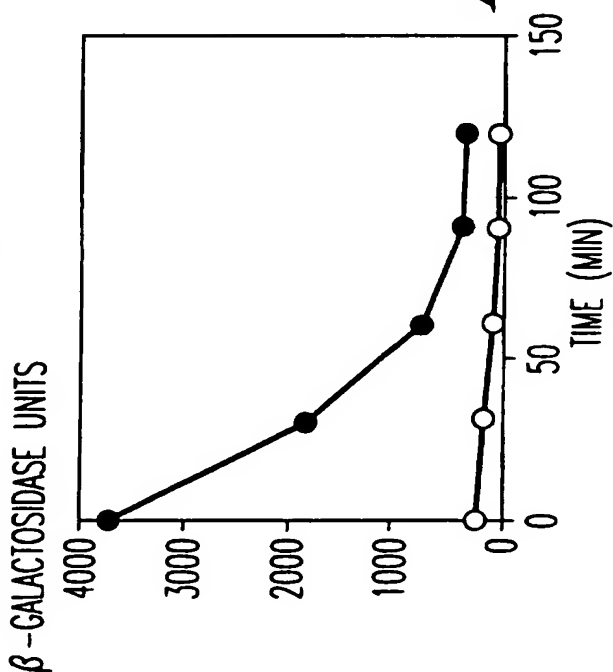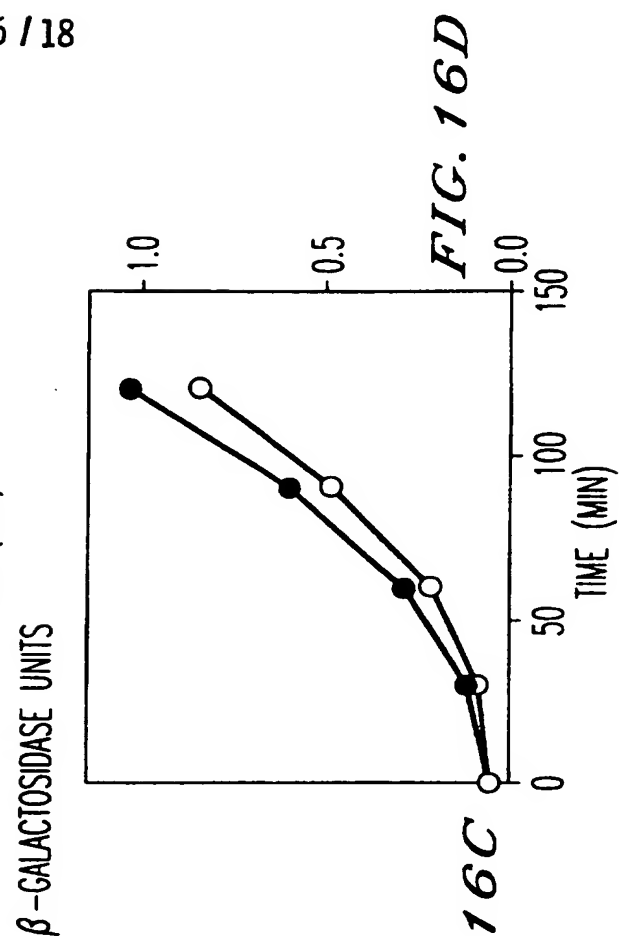   ACAAATGCCAGGACTACTGTCCTGGCAAAAGGTTGGCTAATTAGTATTTATACTTTTTAT

FIG. 13A

S
→ -35
ATTGTTGCATCACCCGCGCCAATGCGTGGCTTAATGCACATCAACGGTTTGACGTACAGACC
                      -10
421 TAACAACGTAGTGGGCGGTTACGCACCGAATTACGTGTAGTTGCCAAACTGCATGTCTGG   480

+1
→

ATTAAAAGCAGTGTAGTAAGGCAAGTCCCTTCAAGAGTTATCGTTGATACCCCTCGTAGTG
481 TAATTTCGTCACATCATTCCGTTCAGGGAAGTTCTCAATAGCAACTATGGGAGCATCAC   540

CACATTCCTTTAACGCTTCAAAATCTGTAAAGCACGCCATATCGCCGAAAGGCACACTTA
541 GTGTAAGGAAATTGCGAAGTTTTAGACATTCGTCCGGTATAGCGGCTTTCCGTGTGAAT   600
↓
S

FIG. 13B

FIG. 15

16 / 18

FIG. 16A

FIG. 16B

FIG. 16C

FIG. 16D

mRNA LEVEL

FIG. 17

-9G ⟶ +1 BamHI

-130 ⊢──────────── -79 -39 ──────── +115
EcoRI
PLASMID pHG309

FIG. 18A

◄─── PL ⟶ ◄─── Plac ⟶
EcoRI ⟶ +1 BamHI

-130 ⊢──────────── -79 -36 ──────── +84
PLASMID pL-plac

FIG. 18B

FIG.19
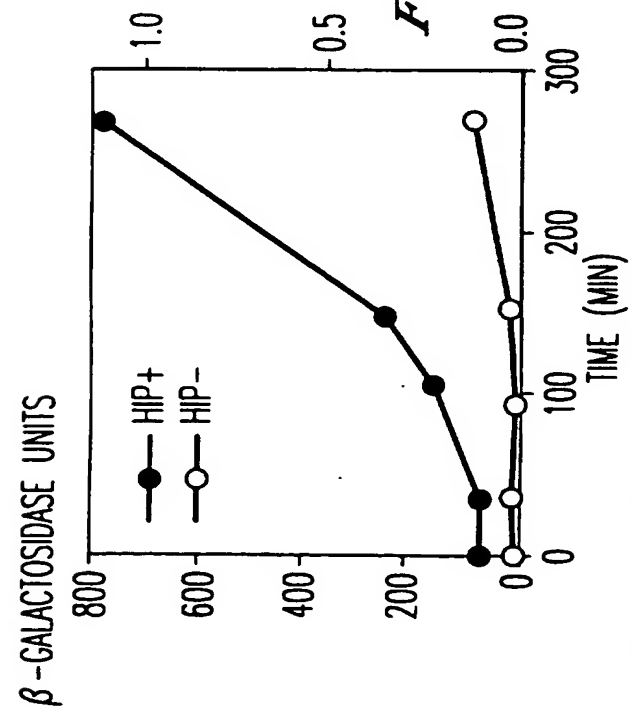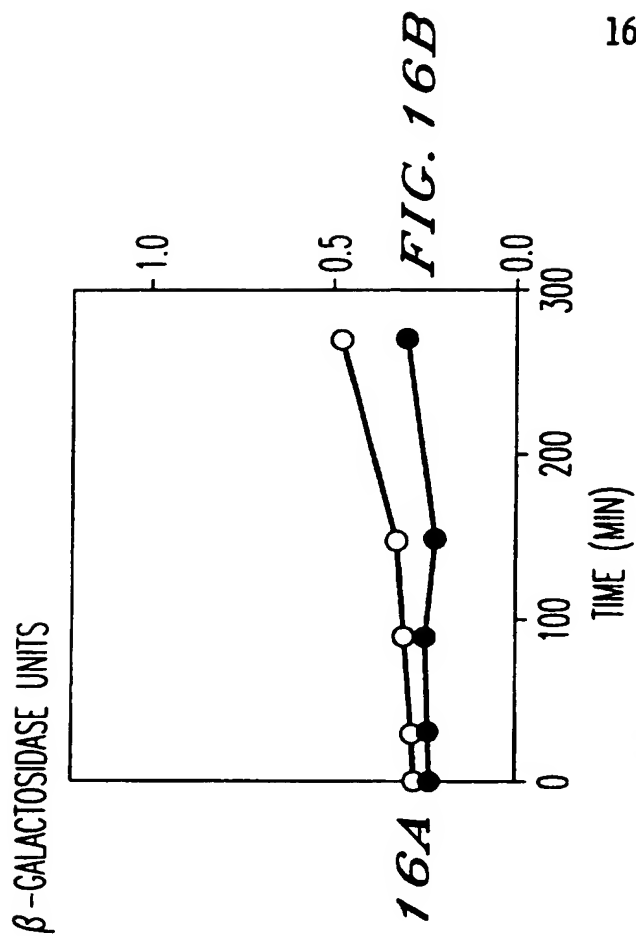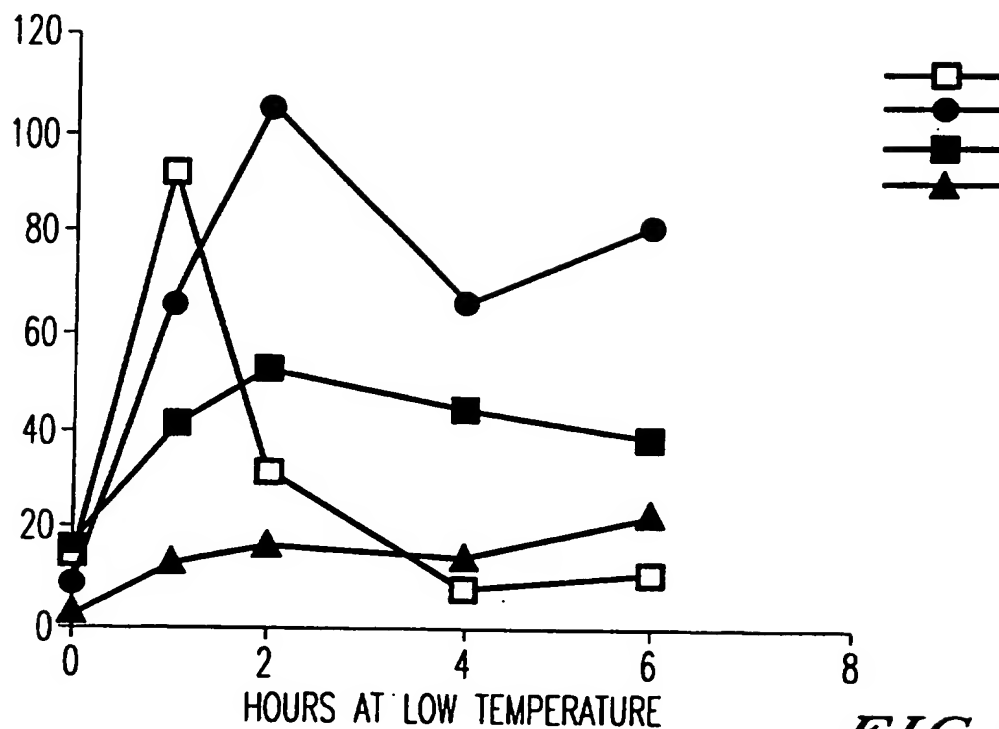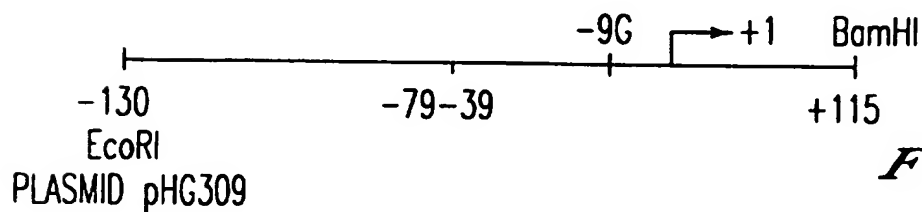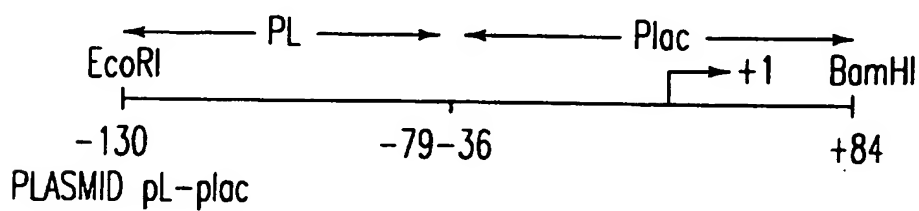
β-GALACTOSIDASE UNITS

PROMOTER FRAGMENT	OVERNIGHT INCUBATION			TEMPORAl COLD SHOCK			mRNA LEVEL
	37C	15C		37C	15C		15C/37C
PcspA +1 +81 lacZ (−449)	5,950	35,730	(6.0)	3,620	8,890	(2.45)	2.8
PcspA +1 +81 lacZ (−209)	5,500	31,860	(5.8)	3,820	9,450	(2.5)	4.2
PcspA +1 +81 lacZ (−59)	2,970	28,170	(9.5)	2,240	8,890	(4.0)	2.5
PcspA +1 +81 lacZ (−40)	1,080	11,500	(10.6)	920	3,640	(4.0)	4.0
PcspA +1 +16 lacZ (−40)	4,180	17,030	(4.1)	1,840	4,920	(2.7)	3.2
PlaoUV5 +1 +3 lacZ (−37)	10,870	11,140	(1.03)	4,670	5,190	(1.1)	1.0
PcspA +1 +81 lacZ (−209)	2,260	47,520	(21)	2,040	14,880	(7.3)	10.0

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :C12P 21/06
US CL   : 435/69.1

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/69.1,320.1; 536/24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Proc. Natl. Acad. Sci. USA, Volume 92, Issued March 1995, Giladi et al., "Enhanced activity of the bacteriophage lambda pL promoter at low temperature", pages 2184-2188, see page 2188, lines 20-23.	36
X	Molecular Microbiology, Volume 11, issued March 1994, Lee et al., "Family of the major cold-shock protein, CspA (CS7.4), of Escherichia coli, whose members show a high sequence similarity with the eukaryotic Y-box binding proteins", pages 833-839, see pages 836-837.	1-6, 8, 10, 14-17, 24-27, 30-35

[X]	Further documents are listed in the continuation of Box C.	[ ]	See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 OCTOBER 1995	15 NOV 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JOHN S. BRUSCA
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	FEBS Letters. Volume 271, issued October 1990, Shirano et al., "Low temperature cultivation of Escherichia coli carrying a rice lipoxygenase L-2 cDNA produces a soluble and active enzyme at a high level", pages 128-130, see pages 128-129.	1-6, 34-35 ------------- 7-39
Y	Journal of Bacteriology, Volume 174, issued June 1992, Tanabe et al., "Identification of the promoter region of the Escherichia coli major cold shock gene, cspA", pages 3867-3873, see pages 3869-3870.	8, 10. 14-17, 24-27, 30-33
Y	Journal of Molecular Biology, Volume 224, issued 1992, Giladi et al., "Supercoiling, integration host factor, and a dual promoter system, participate in the control of the bacteriophage lambda pL promoter", pages 937-948, see page 943.	8, 9, 11-13, 22, 23, 28, 29, 39
Y	Journal of Bacteriology, Volume 173, issued April 1991, Kulakauskas et al., "Efficient introduction of cloned mutant alleles into the Escherichia coli chromosome", pages 2633-2638, see page 2634.	7, 18, 19
Y	Journal of Biological Chemistry, Volume 256, issued February 1981, Horn et al., "The leftward promoter of bacteriophage lambda", pages 2003-2009, see page 2006.	8, 9, 11-13, 18, 22, 23, 28, 29

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

❑ **BLACK BORDERS**

❑ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

❑ **FADED TEXT OR DRAWING**

☑ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

❑ **SKEWED/SLANTED IMAGES**

❑ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

❑ **GRAY SCALE DOCUMENTS**

❑ **LINES OR MARKS ON ORIGINAL DOCUMENT**

❑ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

❑ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.